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Novel phagocytotic hemocytes in the silkworm, *Bombyx mori*

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Abstract: Aim: In order to observe the fine structure of phagocytotic hemocytes with an attention to make sure whether oenonytoids are also phagocytotic in the silkworm, *Bombyx mori*. **Methods:** Larvae of *pnd pS* strain of the silkworm were injected with fluorescent microbeads for this experiment. Stained with acridine orange and propidium iodide, the phagocytotic hemocytes were serially scanned for fine structure by the Radiance 2000 confocal scanning laser microscope (Bio-Rad), and confocal fluorescent images were processed with the Lasersharp 2000 software. **Results:** the observation showed that besides granulocytes and plasmatocytes, some prohemocytes (stem hemocytes) and oenocytoids (prophenoloxidase producer) could also phagocytose fluorescent beads. Furthermore, inside some oenocytoids there were lots of acridine orange green granules that were similar to granules in granulocytes. Spherulocytes were not observed to have phagocytosed any fluorescent beads, but they had large acridine orange green granules. These granules were supposed to be apoptotic bodies which should be introduced by phagocytosis. **Conclusion:** Besides granulocytes and plasmatocytes, the circulating hemocytes oenocytoids are also phagocytotic in the silkworm.

Key words: *Bombyx mori*; hemocytes; phagocytosis; fluorescent microscopy

1 INTRODUCTION

In the silkworm, *Bombyx mori*, there are five types of circulating hemocytes: prohemocyte, granulocyte, spherulocyte, plasmatocyte, and oenocytoid (Nittono, 1960; Akai and Sato, 1973; Beaulaton, 1979; Wago, 1991; Yamashita and Iwabuchi, 2001). Among them, prohemocytes are stem hemocytes; granulocytes are phagocytotic cells; plasmatocytes are in charge of encapsulation; oenocytoids can produce prophenoloxidase (Nittono, 1960; Akai and Sato, 1973; Wago, 1991). No specific function has ever been found with spherulocytes. Recently, a kind of fluorescent method of hemocytes identification has been developed by using acridine orange (AO) and propidium iodide (PI) to stain circulating hemocytes (Ling *et al*., 2003). The different fluorescent characteristics of circulating hemocytes make them to be easily identified at the light-microscope level. In the granulocytes and spherulocytes, there are a lot of AO-positive green granules. Granules of spherulocytes are almost at homogenous size and are larger than those of granulocytes. Granulocyte granules are at heterogenous sizes. These AO-positive granules are supposed to be apoptotic bodies (Wyllie, 1998; Foglieni *et al*., 2001). No AO-positive green granules were found in the prohemocytes and plasmotcytes. Plasmacytes have pseudopods when sticking to the glass cover, but prohemocytes have no pseudopods. Oenocytoids only can be stained by PI and they have red nuclei if observed under fluorescent microscope.

In *Manduca sexta*, granulocytes and plasmatocytes are phagocytotic when there is foreign invasion such as bacteria and fluorescent beads. However, only granulocytes are found to phagocyte apoptotic dead cells *in vivo* in this type of normal larvae (Ling and Yu, 2005). Many more types of circulating hemocytes such as granulocytes and plasmatocytes in the silkworm are phagocytotic, especially some prohemocytes can also phagocytose tissue debris (Ling *et al*., 2005). But we don't know weather oenocytoids and spherulocytes are also phagocytotic. According to the AO-positive granules in spherulocytes, this type of hemocytes might be able to phagocytose foreigners. In this study, we used confocal scanning fluorescent microscope to observe the fine structure of phagocytotic hemocytes with an attention to make sure whether oenonytoids are also phagocytotic.

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2 MATERIALS AND METHODS

2.1 Experimental animals

The *pnd pS* strain of the silkworm, *Bombyx mori*, was used for this experiment. Larvae were reared on an artificial diet and kept at 25℃ under a 16L : 8D photocycle. Larval age was given in days described by Kiguchi and Agui (1981) for larval molting stage and Kiguchi *et al*. (1985) for the fifth instar and metamorphic stages. Day 0 indicates the day when larval ecdysis occurs. Most of the fifth instar larvae begin to wander on day 5.

2.2 Acridine orange and propidium iodide staining

To maintain the physiological environment of hemocytes, larval insect saline (130 mmol/L NaCl, 5.0 mmol/L KCl, and 1.0 mmol/L $CaCl_2$; pH 7.0) was used to dissolve acridine orange and propidium iodide. To observe circulating hemocytes, hemocytes were stained according to Ling *et al*. (2003). To scan and image phagocytotic hemocytes, the Radiance 2000 confocal scanning laser microscope (Bio-Rad) was used. Confocal fluorescent images were processed with the Lasersharp 2000 software. Sometimes hemocytes were also viewed using a differential interference fluorescence microscope (Nikon Eclipse E600 System).

2.3 Phagocytosis of foreign matters

For better understanding of the phagocytic function, about 9×10^6 fluorescent microbeads [FluoSpheres Polystyrene Microspheres for Tracer Studies, 1.0 μm, Red (580/605); Molecular Probes] per gram larval body weight were injected into each larva (V-2) with a microliter syringe (25 μL; Hamilton) in order to allot approximately five to ten fluorescent microbeads to each circulating hemocyte. After about 6 h, when all free fluorescent microbeads had disappeared from the hemolymph, circulating hemocytes were observed by acridine orange and propidium iodide staining as described before.

3 RESULTS AND DISCUSSION

Following staining with AO and PI, we found some circulating prohemocytes having phagocytosed fluorescent beads. The serially scanned prohemocytes clearly showed that the fluorescent microbeads were already introduced into the cell by means of phagocytosis (Fig. 1). This prohemocyte has a clear nucleus in which there is a green granule as shown by Ling *et al*. (2003). In the silkworm hematopoietic organs, some isolated prohemocytes were able to phagocyte cell and tissue debris, by which to transform into the morphology of granulocytes (Ling *et al*., 2005).

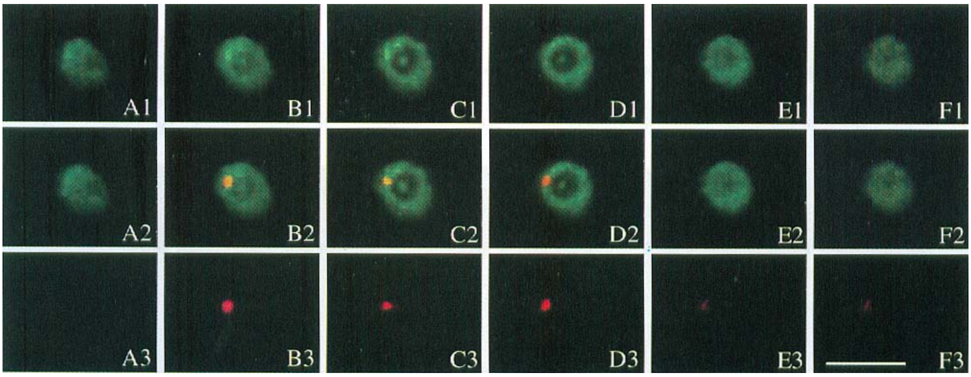

Fig. 1 Morphology of a prohemocyte that had phagocytosed fluorescent beads

This hemocyte was serially scanned under the Radiance 2000 confocal scanning laser microscope with a step of 2 μm. Pictures were imaged with green (A1 – F1) and red (A3 – F3) filters. Pictures in the middle (A2 – F2) were combined from both. Scale bar: 20 μm.

Granulocytes were phagocytotic as reported very early (Akai and Sato, 1973). In the scanned images of granulocytes, the fluorescent beads existed among green granules (Fig. 2). However, the phagocytosed beads by this type of hemocytes were less than by plasmatocytes (Fig. 3). There was the same situation with *M*. *sexta* (Ling and Yu, 2005).

By examining lots of circulating hemocytes, we finally found some oenocytoids that were really phagocytotic. We scanned the target oenocytoid in Fig. 4 and found that a fluorescent bead had already been phagocytosed. In this oenocytoid, there were also many green granules stained by AO. Since AO-positive green granules were supposed to be apoptotic bodies (Wyllie, 1998; Foglieni *et al*., 2001), it was possible that this oenocytoid had already phagocytosed some dead cells before. The fine structure in Fig. 4 supported that some oenocytoids were actually phagocytotic.

As to spherulocytes, they had not been found to be phagocytotic. Here we presented serially scanned images of one spherulocyte (Fig. 5). The spherulocytes contained many homogenous green granules that were

AO-positive. Again we still wonder whether this type of hemocytes has the phagocytotic function because these green granules should be apoptotic bodies that can be stained by AO (Wyllie, 1998; Foglieni *et al*., 2001). We did not find this type of hemocyte that could phagocytose any fluorescent bead. But the AO-positive granules suggest that they could be phagocytosed apoptotic bodies (Wyllie, 1998; Foglieni *et al*., 2001).

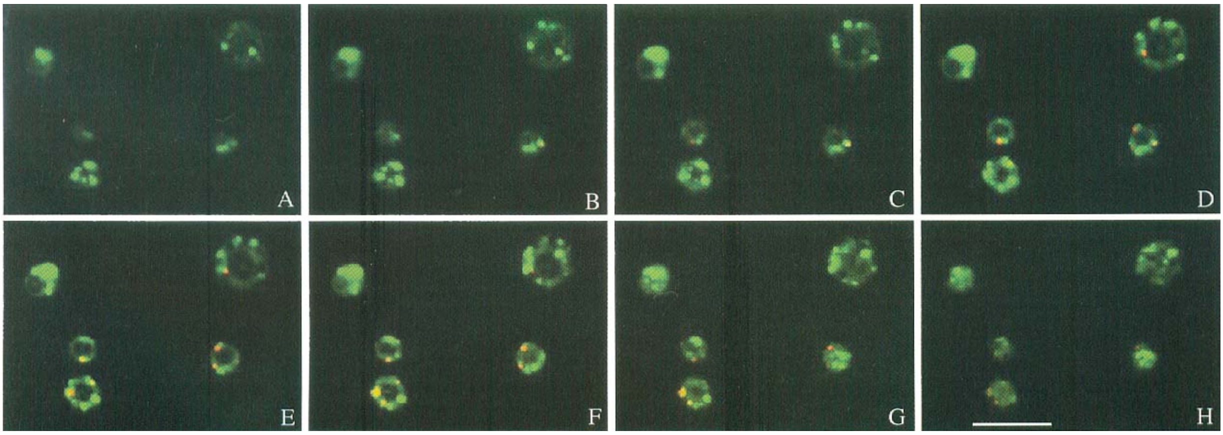

Fig. 1 Morphology of phagocytotic granulocytes

Pictures (A – H) were imaged as above with a step of 1 μm and combined from pictures imaged with green and red filters. In this granulocyte, the phagocytosed fluorescent beads existed among the heterogenous green granules. Scale bar: 20 μm.

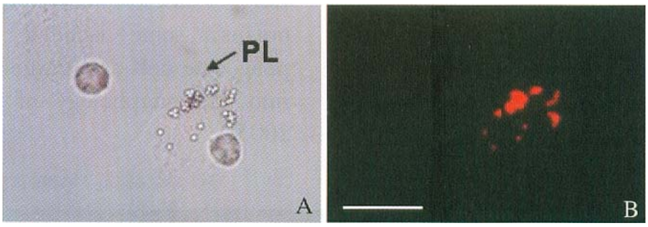

Fig. 3 Morphology of a plasmatocyte that had already phagocytosed a lot of fluorescent beads

Hemocytes were imaged under the Nikon Eclipse E600 System. The right picture (B) was imaged using red filter, and the left one (A) was under light microscope. PL: plasmatocyte. Scale bar: 20 μm.

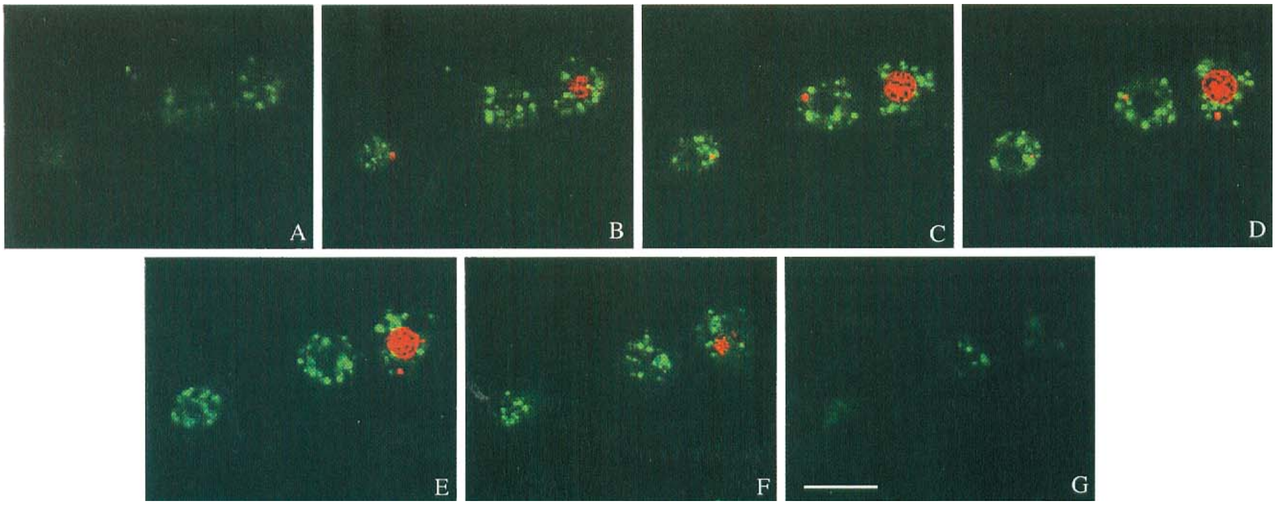

Fig. 4 Morphology of a phagocytotic oenocytoid

These hemocytes were serially scanned under the Radiance 2000 confocal scanning laser microscope with a step of 2 μm, and pictures (A – G) were combined from those imaged with green and red filters. In this oenocytoid, there was a PI-positive red nucleus that was the typical characteristics of oenocytoids. In the cell, there were a lot of green granules that were stained green by AO, and there was also a phagocyted fluorescent bead. Scale bar: 20 μm.

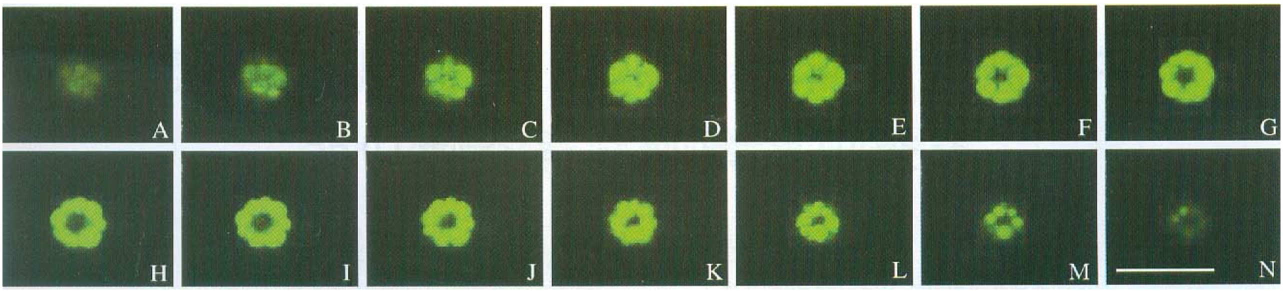

Fig. 5 Morphology of a spherulocyte

The hemocyte was serially scanned under the Radiance 2000 confocal scanning laser microscope with a step of 1 μm by using green filter（A-N）. Scale bar：20 μm.

In most insects，granulocytes and/or plasmatocytes are phagocytotic（Lackie，1988；Wago，1991；Lavine and Strand，2002）. But in the silkworm，besides granulocytes and plasmatocytes，some prohemocytes and oenocytoids are also able to phagocytose fluorescent beads. Prohemocytes are stem hemocytes and oenocytoids，the place for prophenoloxidase production（Nittono，1960；Akai and Sato，1973；Beaulaton，1979；Wago，1991；Yamashita and Iwabuchi，2001）. We have no idea what factors there are that can incite these two types of hemocytes into phagocytosis. In future we want to make clear the mechanism because this novel phagocytosis could help insects to fight against foreigners into their bodies effectively.

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家蚕拟浆细胞具有吞噬功能

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摘要：目的 通过观察家蚕 *Bombyx mori* 吞噬细胞的微细结构,来确定拟绛色细胞是否也具有吞噬功能。方法 :用荧光小球微量注射家蚕 *pnd pS* 品系的幼虫,经荧光染色剂丫啶橙和碘化丙啶染色循环血细胞后,在荧光显微镜下观察并扫描拍摄。结果 观察发现除颗粒细胞和浆血细胞外,一些原血球细胞(血干细胞)和拟绛色细胞(多酚氧化酶)也能吞噬荧光小球。在拟绛色细胞里还发现许多和颗粒细胞一样的能被丫啶橙染色的颗粒。尽管在小球细胞中没有发现被吞噬的荧光小球,但该类血球有比较多的能被丫啶橙染色的大颗粒,这表明它们可能是已经被吞噬的凋亡小体。结论 :除颗粒细胞和浆血细胞外,一些原血球细胞和拟绛色细胞也能吞噬荧光小球。说明拟绛色细胞也具有吞噬功能。

关键词：家蚕;血细胞;吞噬;荧光显微镜

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